SERUM LEVELS OF C-REACTIVE PROTEIN AND ANTI-MULLERIAN HORMONE, FOLLICLE-STIMULATING HORMONE, AND INHIBIN-B IN WOMEN ATTEMPTING PREGNANCY IN NORTH CAROLINA

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A thesis submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Arts in the Department of Anthropology.

Chapel Hill

2021

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ABSTRACT

Anneliese M. Long: Serum Levels of C-Reactive Protein and Anti-Mullerian Hormone, Follicle-Stimulating Hormone, and Inhibin-B in Women Attempting Pregnancy in North Carolina
(Under the direction of Amanda L. Thompson)

The present study sought to investigate the relationship between of ovarian reserve and immune function. Using data from a subset of 790 nonpregnant, cycling women who were attempting pregnancy between the ages of 30 and 44 participating in the Time to Conceive Study from 2008 to 2016, a prospective observational cohort study in central North Carolina, this project aimed to test the relationship between chronic inflammation on ovarian reserve. We use three biomarkers of ovarian reserve, FSH, inhibin-b, and AMH, a biomarker of chronic inflammation, CRP, and self-reported questionnaire data to test the association between chronic inflammation and ovarian reserve. Multivariate regression models were employed, adjusting for other factors that influence ovarian reserve. The results from this exploratory investigation suggest an association between CRP and inhibin-b, and CRP and AMH when CRP levels are above 3 mg/L.

ACKNOWLEDGEMENTS

I thank my committee, Dr. Amanda Thompson, Dr. Mark Sorensen, and Dr. Paul Leslie for their insight and feedback revising this thesis. I am also grateful for the National Institute of Environmental Health Sciences (NIEHS) for training and feedback, particularly from Dr. Anne Marie Jukic and Dr. Johanna Jahnke for their ongoing mentorship.

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LIST OF ABBREVIATIONS

AFC	Antral Follicle Count
AMH	Anti-Mullerian Hormone
ART	Assisted reproductive technology
BMI	Body Mass Index
CRP	C-Reactive Protein
DES	Diethylstilbestrol
DOHaD	Developmental Origins of Health and Disease
DOR	Diminished Ovarian Reserve
FOR	Functional Ovarian Reserve
FSH	Follicular Stimulating Hormone
HIV	Human Immunodeficiency Virus
HPA	Hypothalamic-Pituitary-Adrenal
hsCRP	High-Sensitivity C-Reactive Protein
IVF	In-Vitro Fertilization
LH	Luteinizing Hormone
MIS	Mullerian Inhibiting Substance
NGF	Non-Growing Follicles

NM	Natural Menopause
OR	Ovarian Reserve
POA	Premature Ovarian Aging
POF	Premature Ovarian Failure
POI	Premature Ovarian Insufficiency
TOR	Total Ovarian Reserve
TEE	Total Energy Expenditure

CHAPTER 1: INTRODUCTION

The connection between systemic chronic inflammation and ovarian reserve remains relatively unexplored in the field of human reproductive biology. Total ovarian reserve (TOR) is defined as the complete follicle pool of an individual, representing the number of viable oocytes and the functional potential of the ovary over time (Gleicher et al., 2011). Systemic chronic inflammation refers to the long-term activation of the innate immune system (Ridker, 2003a). The appropriate function of the immune system and its inflammatory response is vital to the normal ovarian cycle and the production of a viable germ cell by the human female for potential fertilization (Boots & Jungheim, 2015). Conversely, inflammatory responses that deviate from those associated with ovulation, particularly during the follicular phase of the menstrual cycle, are associated with the disruption of ovarian function (Boots & Jungheim, 2015).

In viewing the association between systemic chronic inflammation and total ovarian reserve, one must also acknowledge that human biology is inevitably shaped by the experiences and exposures an individual has accumulated across the life span (Kuh et al., 2003). The age-related decline in ovarian function, in both its quality and quantity of oocytes, across the life course is synonymous to ovarian aging, which eventually reflects the end of reproductive capacity in individuals with ovaries (Gleicher et al., 2011). One would expect that sustained chronic inflammation, which can acutely disrupt ovarian function, would also have an impact on the functional potential of the ovary over time and therefore be reflected in ovarian reserve status (Weiss et al., 2009).

The factors mediating the ovarian aging process in humans and other mammals have remained largely ambiguous. Findings from an animal model have demonstrated a connection between the decline in follicles and chronic inflammation, suggesting inflammation is a mechanism of reproductive aging in mice (Lliberos et al., 2021). The decrease in follicle numbers over the reproductive life span in female mice corresponded to an increase in several inflammatory markers in serum and in the intra-ovarian environment (Lliberos et al., 2021). These findings suggest systemic inflammation may be involved in the ovarian aging process in humans as well, which is characterized by the eventual depletion of follicles of sufficient quality to lead to pregnancy (Zhang et al., 2019). Chronic inflammation may serve as one mechanism underlying the natural reproductive aging process and further explain variation in ovarian reserve through different pacing of the ovarian aging process towards natural menopause (Zhang et al., 2019; Broekmans et al., 2007). Previous studies on the relationship between certain inflammatory conditions, including pelvic inflammatory disease and Crohn's disease, and lower age-matched ovarian reserve status support inflammation as playing a key role in increasing the rate of follicle depletion (Cui et al., 2016; Freour et al., 2012; Kitajima et al., 2011).

Life history theory is a useful framework to consider in the connection between reproductive and immune function given the allocation of energy may involve trade-offs between different bodily systems based on the present and the developmental environment (Vitzthum, 2009; Ellison, 1990; Voland, 1998). Further, the Developmental Origins of Health and Disease (DOHaD) hypothesis remains an important framework given the role early environments serve in shaping adult outcomes and phenotypes.

This study examines the relationship between low-level chronic inflammation and ovarian reserve using biomarker data from 843 cycling women between the ages of 30 and 44

participating in the Time to Conceive Study, a prospective observational cohort study of women attempting pregnancy in the Chapel Hill region of North Carolina. The data were collected from 2008 to 2016, with biomarker data collection beginning in 2012. Three biomarkers of ovarian reserve are examined- follicle-stimulating hormone (FSH), inhibin-b, and Anti-Mullerian hormone (AMH) along with high sensitivity C-reactive Protein (hsCRP), in combination with self-reported questionnaire data to determine the relationship between systemic chronic inflammation and ovarian reserve status. The following research question will be addressed with these data:

- Are serum levels of high-sensitivity C-reactive protein (hsCRP) related to serum levels of follicle stimulating hormone (FSH), inhibin-b and Anti-Mullerian hormone (AMH)?

Given previous research suggesting that chronic, systemic inflammation is associated with follicle depletion, which will be measured in this analysis through serum levels of AMH, it is hypothesized that chronic inflammation is negatively associated with ovarian reserve. The disruption of follicle growth and regulation remain the key underlying reasoning for the existence of a relationship between ovarian reserve and chronic inflammation, which animal models have previously investigated. Several biological pathways may lie beneath the relationship between chronic and inflammation and ovarian reserve, including differential pacing of ovarian aging within and between populations that may be measurable through AMH. The findings of this study could suggest avenues for future research in reproductive aging and menopause.

CHAPTER 2: BACKGROUND

Part I: Chronic Inflammation and Immune Activation

Ovulation and Inflammation

To understand how chronic, low-grade inflammation may disrupt follicle development over time, the potential role of inflammation in normal ovarian physiology needs investigation. Ovulation takes place in mammalian reproduction in two distinct parts of the menstrual cycle: folliculogenesis and luteinization (Boots & Jungheim, 2015). The inflammatory process includes direct and indirect signals that induce several biological processes that are also seen in the process of ovulation (Boots and Jungheim, 2015). For ovulation to happen, the ovarian tissue ruptures to expel the mature oocyte, which is set in motion by inflammation that weakens the follicle wall (Epsey, 1994). In the ovary, there are five layers of tissue enveloping each oocyte: the epithelium, the tunica albuginea, the theca externa, the theca interna, and the granulosa (Epsey, 1994). As the dominant follicle continues to grow, the thecal layers come together with the tunica albuginea layer, and thin to allow for the eventual rupture (Boots & Jungheim, 2015). Luteinizing hormone (LH), surges prior to ovulation in response to estrogen produced in the ovaries, and there is a subsequent release of histamines and other inflammatory mediators as part of the process of ovulation (Boots & Jungheim, 2015). However, the element of timing related to inflammation is important. Only briefly before and at ovulation are inflammatory markers thought to be part of normal ovarian physiology (Boots & Jungheim, 2015). Therefore, the successful generation of follicles and a dominant, mature follicle for ovulation require a normal

inflammatory response, and deviation from this normal response may affect ovarian reserve or the markers of ovarian reserve.

C-Reactive Protein and Chronic Inflammation

Chronic inflammation, as measured through a specific biomarker, c-reactive protein, is associated with metabolic and cardiovascular disease (Ridker, 2003a). C-reactive protein (CRP) plays a key role in the activation of the innate immune response and has emerged in the past two decades as a predictor of future adverse cardiovascular events and therefore is frequently used as a clinical screening tool (Ridker, 2003a; Ridker, 2007). CRP is an acute-phase reactant and a marker of systemic inflammation (Boots & Jungheim, 2015). The acute-phase response includes the nonspecific biochemical and physiological result of most tissue damage in its various forms, including infection and inflammation (Pepys & Hirschfield, 2003). A number of other pro-inflammatory proteins are upregulated in hepatocytes in response to tissue damage (Pepys & Hirschfield, 2003). Research on the inflammatory response has found that metabolic stress and dysfunction can also create a chronic, low-grade inflammatory state (Kushner & Antonelll, 2015). Therefore, there are ongoing debates as to whether CRP is causal, or simply represents one outcome in a cascade of biological processes leading to adverse vascular events (Kushner & Antonelll, 2015). However, in either case, CRP has a clear association with adverse vascular events, and perhaps other health outcomes extending into reproductive function.

Although C-reactive protein is well-documented to be associated with the inflammatory response, the normal variation of the acute phase reactant has remained an ongoing dispute. Decades ago, investigators proposed that levels of CRP greater than 10mg/L indicated current infection, a significant inflammatory process, while more recently it has been proposed that levels greater than 3/mg/L serve as an indicator of high risk for cardiovascular events (Kushner

& Antonelll, 2015). Generally, measures above 3 mg/L appear to represent a minor elevation of CRP, relatively speaking, compared to the immune response to infection and other more acute functions of the immune system (Kushner & Antonelll, 2015).

In relation to infectious disease, C-reactive protein has been demonstrated to increase up to 1,000-fold in response to some bacterial infections (Sproston & Ashworth, 2018). Plasma levels beginning at 1 μg/mL can increase to levels exceeding 500 μg/mL within one to three days after severe tissue damage (Sproston & Ashworth, 2018). However, CRP levels decrease exponentially over 18 to 20 hours after the stimuli stop, which resembles the known half-life of CRP (Sproston & Ashworth, 2018). Additionally, it is important to note that the underlying trigger for the inflammatory response, including CRP, are the signals presented by distressed or injured cells, meaning it is itself a consequence of other insults on or in the body (Kushner et al., 2006).

The expression of chronic inflammation represents a mosaic of the lived environment, including developmental and present conditions and bodily states. Higher levels of CRP have also been associated with environmental irritants, obesity, genetics, age, and several other factors (Kushner et al., 2006). Environmental and industrial toxicants remain an active area of research in considering the cause of chronic inflammation. Chemicals including phthalates, bisphenols, and flame retardants appear to change the signaling pathways underlying the inflammatory response (Furman et al., 2019). Overall, multiple levels of exposure and predisposition exist that promote the expression of systemic chronic inflammation across the life course, which likely underlies the association between CRP and cardiovascular risk (Furman et al., 2019). Early environments also likely play a large role in “priming” the immune system and pathways of inflammation, which may also explain the association between adverse health conditions,

particularly vascular events, and chronic inflammation in industrialized contexts (McDade, 2012).

Follicular Dynamics and Chronic Inflammation

There is evidence that low-chronic and systemic inflammation interacts and affects follicle development, particularly in ovarian aging (Yang et al., 2020). In mice, an association has been demonstrated between several pro-inflammatory cytokines, including IL-1α/β, TNF-α, IL-6, which are secreted by immune cells, and follicle depletion as female mice age (Lliberos et al., 2021). Inflammaging, which describes the chronic low-grade and sterile inflammation that advances with age, may explain this relationship between chronic inflammation and follicle depletion (Franceschi and Campisi, 2014; Lliberos et al., 2021). Further, several immune-related genes have been found to be upregulated in the ovaries of mice of older age, again suggesting a relationship between ovarian aging and inflammation over the life course (Zhang et al., 2020). The underlying mechanisms connecting chronic inflammation to follicle dynamics and their depletion in humans are not well-understood, but oxidative stress in the form of excess reactive oxygen species (ROS), has been suggested as one causal pathway (Yang et al., 2020).

The normal process of follicle development over the reproductive life span may be disrupted through chronic inflammation through an altered follicular environment. Women with reduced ovarian reserve or advanced age have altered follicular environments as compared to other women (Pacella et al., 2012). Though yet to be demonstrated in the context of chronic inflammation, it is probable that the micro-environment of the ovary would reflect the larger environment of the body if it were chronically inflamed. Evidence supporting the association between the ovarian reserve and chronic inflammation primarily comes from studies on women with autoimmune conditions and measurement of their associated ovarian reserve. Several

autoimmune conditions have been associated with lower AMH levels, including rheumatoid arthritis, Behçet's disease, and spondyloarthritis (Henes et al., 2015). Chronic pelvic inflammation, Crohn's disease, and endometriosis have also each been separately associated with lower AMH levels (Cui et al., 2016; Freour et al., 2012; Kitajima et al., 2011). While a direct connection between chronic inflammation and ovarian reserve remains elusive, the evidence to date appears to support a relationship between ovarian aging and chronic systemic inflammation.

Though chronic systemic inflammation does preliminarily appear to be disruptive to normal ovarian function related to follicle recruitment and development, based on current evidence, it is noteworthy that an appropriate and adequate inflammatory response is still required for normal ovarian function (Boots & Jungheim, 2015). Ovulation itself is an inflammatory process; therefore, it would be lack of an appropriate inflammatory response that could disrupt ovarian function rather than the inflammatory response alone (Boots & Jungheim, 2015). Pro-inflammatory states that exist outside of the range of normal variation may have a negative influence on ovarian follicle dynamics, which may explain the relationship between inflammation, ovarian reserve, and obesity (Boots & Jungheim, 2015). Adiposity has been associated with a pro-inflammatory response, which may help explain the relationship between obesity and anovulation (Giviziez et al., 2016; Schäffler et al., 2006).

Follicular wave dynamics, which represent the developmental timing and tendencies of follicle development, has been directly studied in the context of chronic inflammation. Systematic inflammation, as measured by high-sensitivity c-reactive protein (hsCRP), has been associated with follicular wave dynamics (Clancy et al., 2013). In their study of twenty-five Polish women, Clancy and colleagues found that women with three waves had higher average log hsCRP concentrations, as compared to those with two waves (2013). This study suggests that

those that have overall more follicular waves, which are characterized as the synchronous growth of a group of follicles that is then followed by the "selection" of a follicle for continued growth with the regression of all other follicles in that wave, is associated with higher levels of systemic inflammation (Clancy et al., 2013). The association between three waves and higher CRP was hypothesized to result from the process of tissue remodeling occurring with each wave, which would be presumably higher in those undergoing multiple waves of follicular development (Clancy et al., 2013). The number of follicular waves is known to vary significantly, and this could correspond to ovarian reserve status and AMH levels. Overall, chronic, systematic inflammation is associated with lower AMH levels, which may in part be explained through differences in follicular development, potentially through the dysregulation of pro-inflammatory cytokines leading to follicle death (Lliberos et al., 2021).

Part II: Ovarian Reserve

Introduction to Ovarian Reserve

The measurement of fecundability, the probability of a successful pregnancy over a menstrual cycle, and fecundity, the reproductive capacity of an individual more broadly, remain important areas of research for clinical, scientific, and epidemiological inquiry into human reproduction (Baird & Steiner, 2011). However, one of the ongoing methodological difficulties remains in the study of fecundability and fecundity: How to measure these in the absence of the desire or opportunity to conceive?

Epidemiological studies have often investigated the subject of fecundity through time-to-conception or time-to-pregnancy approaches to determine the fecundability of individuals over the course of the menstrual cycle (Baird & Steiner, 2011). Through following individuals attempting pregnancy over time, until the end of the investigation or the outcome has occurred,

the collective knowledge of conception, pregnancy, and fecundity has greatly increased over the past decades (Baird, 1988; Baird et al., 1986; Joffe, 1997). Nonetheless, the ongoing issue of selection bias remains a point of methodological difficulty due to the time-to-pregnancy metric necessitating research subjects to plan their pregnancy. Measuring the probability of pregnancy in a given cycle therefore is difficult to assess directly since the prerequisite would be attempting pregnancy in the first place (Bonde et al., 2006; Weinberg et al., 1994).

Indirect measures of fecundity and fecundability are therefore an active area of research to bridge the gap, and to address the issue of selection bias through emerging tools, particularly through integrating biomarkers into studies on human fecundity and fecundability (van Rooij et al., 2002). Anti-Mullerian hormone (AMH), also referred to in the literature as Mullerian-inhibiting substance (MIS), has emerged as a promising measurement of ovarian reserve (van Rooij et al., 2002). Therefore, the measurement of ovarian reserve, relative to age-matched comparisons, could represent one measurable aspect of fecundity for epidemiological and scientific investigation. Yet, AMH represents only one of several markers of ovarian reserve.

Other biomarkers and measurements of the ovarian reserve include follicle-stimulating hormone, inhibin-b, oestradiol, antral follicle count, ovarian volume, challenge tests, and ovarian biopsy (Maheshwari et al., 2006). Nevertheless, many of these measures have significant limitations, particularly in their predictive value and level of invasiveness, when used alone. For instance, there is a large inter-cycle variability of follicle-stimulating hormone (FSH). Antral follicle count (AFC) refers to the number of follicles in the tertiary stage of development during the early follicular phase, as determined by their size (Broekmans et al., 2010).

Ovarian biopsy remains an invasive and poorly understood method for predicting pregnancy. It is important to note that antral follicle count remains another important

measurement tool for predicting ovarian reserve and fecundity and often serves as a point of comparison for other methods, though it must be measured through transvaginal ultrasound rather than through serum (Maheshwari et al., 2006). Primordial follicles, the earliest and "resting" stage of development of oocytes, are also a marker of fecundity given this refers to the total number of follicles in the ovary, hence these are the entire ovarian reserve (Broekmans et al., 2010). However, primordial follicles are hard to measure given their small size, among other methodological difficulties.

In a study that measured AFC, AMH, FSH, inhibin-b, and ovarian primordial follicle count, AMH was significantly correlated with AFC and ovarian primordial follicle count after adjusting for age (Hansen et al., 2011). Similar studies in humans and non-human mammals have demonstrated that levels of AMH provide an approximation of the number of early growing follicles that are between, 0.05–2.00 mm in diameter (Visser et al., 2006; Kevenaar et al., 2006). This correlation corresponds with current understandings of the production of anti-müllerian hormone (AMH). AMH is part of the transforming growth factor β family of growth, and within the ovary, the hormone has an inhibitory action on follicle recruitment and on the responsiveness of growing follicles to follicle-stimulating hormone (FSH) (Visser et al., 2006). Therefore, it is intimately connected to the regulation of follicle development and recruitment, which is presumed to be intertwined with the total primordial follicle pool, or the ovarian reserve. This has been further supported by a study that preformed histological primordial follicle counts and compared the count to AMH and AFC, which were found to be correlated to primordial follicle count (Hansen et al., 2007). Inhibin-b is also a potential marker of ovarian reserve released by small antral follicles early in development (Robertson, 2012). As well, for inhibin-b there is a negative feedback mechanism with FSH, in which inhibin-b communicates with the pituitary

gland to decrease FSH production (Robertson, 2012). But, based on current literature, it is unclear exactly how inhibin-b relates to ovarian reserve over the life course (Robertson, 2012). AMH has been established as a marker of the number of early growing follicles and antral follicles, the connection between AMH, and more broadly ovarian reserve, and recorded fertility remains somewhat ambiguous. Oocyte quality may play a significant role in a successful pregnancy outcome, which is in essence harder to quantify and measure in any given menstrual cycle. Future research is needed to further elucidate the relationship between fertility outcomes and ovarian reserve measures.

Ovarian Aging

Ovarian aging is closely interconnected to the primordial follicle pool given the role of ovarian reserve in the timing of menopause. Age at menopause and the endocrine changes occurring in the year prior to menopause are understood to be a direct result of follicle depletion (te Velde et al., 1998). Menopause is defined as the cessation of menstruation and ovulation, reflecting the loss of ovarian follicles at the end of the reproductive lifespan (Gold, 2011). Clinically, the cessation of menstrual cycles for one year is the recommended measurement of the timing of menopause, as the World Health Organization suggests (Gold, 2011). However, it is important to note that this measurement of the timing of menopause does not reflect endocrine changes directly, but rather uses an indirect method to infer the underlying endocrine changes occurring in the body (Gold, 2011). Based on cross-sectional studies, the median age at menopause of white women residing in industrialized contexts is between 50 and 52 years old (Gold, 2011).

Menopause is considered a natural component of human life history and represents a common experience of all human females. The cessation of reproductive capacity in humans'

decades prior to the end of the life span is a unique feature that has not been observed in any of our closest primate relatives (Sievert, 2014). Several evolutionary theories have been proposed to explain its existence in the human life course, including the extended caretaking model required to rear human children (Austad, 1994). Though the existence of natural menopause is universal in human females, there does appear to be a level of variation in the age at menopause across populations (Sievert, 2014). Some studies have indicated that socioeconomic and racial and ethnic differences exist in the age at menopause (Sievert; 2014; Gold et al., 2001; Gold, 2011; Pelosi et al., 2015). These differences could be related to both adult and developmental exposures interacting with the endocrine system, and more specifically, follicle development.

Given the relationship between decreasing follicle numbers and the depletion of the ovarian reserve and the timing of menopause, AMH is a promising predictor of the menopausal transition (van Rooij et al., 2004; Finkelstein et al., 2020; Bertone-Johnson et al., 2018). It is thus important to reflect on the relationship between ovarian reserve through the reproductive life course, with menopause marking the cessation of fecundity. Variability in ovarian aging, mirrored by the decrease in ovarian reserve, has the potential to explain not only the mechanisms underlying the age at menopause, but also the mechanisms underlying decreases in fecundity and fertility earlier in the reproductive life span in the decades prior to the menopausal transition (Broekmans et al., 2009).

The trajectory of reproductive aging within the ovary begins well before birth. At about 4 months of gestation, the ovaries contain on average between 6 to 7 million oocytes, which are each surrounded by a layer of granulosa cells that form the primordial follicle pool (Broekmans et al., 2009). The vast majority of these oocytes are lost prior to birth, after which 1 to 2 million primordial follicles remain and the subsequent rate of loss slows down (Broekmans et al., 2009).

At menarche, at least 300,000 remain from the initial pool of millions during fetal development, and by menopause, the number has generally dropped below 1,000 (Broekmans et al., 2009). A model of ovarian reserve, quantified by non-growing follicles (NGF), has been developed through synthesizing eight quantitative histological studies of the human ovary at known ages and has suggested approximately 81% of the variance in the number of NGF is due to chronological age (Wallace & Kelsey, 2011). However, some studies have proposed that the rate of loss may vary by age, with the pace of small follicle loss accelerating during the late thirties, suggesting that there is not a completely static rate of follicle loss through the life course (Gougeon et al., 1994). However, the rate of follicle loss and pace of ovarian aging is an ongoing debate among reproductive biologists (Hansen et al., 2008).

Further, differences in the distribution of follicle size may influence observed AMH levels. Smaller follicles have been demonstrated to decrease in number over the course of reproductive aging, while larger follicles increase (Bentzen et al., 2013). This finding was coupled with lower serum AMH levels correlated to higher chronological age and lower AFC. Given that AMH is produced by small antral follicles, it is unsurprising that decreasing numbers of small follicles are associated with lower serum levels of AMH (Bentzen et al., 2013). In mice, serum AMH levels have been strongly correlated to primordial follicle pools, further supporting the association between the biomarker and ovarian aging in non-human mammals (Kevenaar et al., 2006). Additionally supporting the connection between AMH levels and ovarian aging is the strong correlation between AMH levels and AFC in a study on ovarian aging, even as the other markers of ovarian reserve did not change to reflect ovarian aging observed through decreases in AFC (de Vet et al., 2002). Further, while AMH and AFC have not been demonstrated to reflect the quality of the ovarian reserve and oocytes, the decline in quantity has been hypothesized to

impact quality (Vollenhoven & Hunt, 2018; Broekmans et al., 2009). Genetic variation may have explained some differences in ovarian reserve, particularly through the FMR1 gene, which has been implicated in the speed of follicular recruitment (Gleicher et al., 2011).

Overall, the variability in ovarian aging that cannot be explained by chronological age alone remains an active area of research (Broekmans et al., 2007). A validated mathematical model of AMH, developed from conception to menopause from 3,260 healthy pre-menopausal females, has demonstrated that 34% of the variation in serum levels is due to age alone (Kelsey et al., 2011). This study further observed that the peak AMH level occurred at 24.5 years old, with a neonatal peak and potentially a pre-pubescent peak (Kelsey et al., 2011). The measurement of anti-müllerian hormone and its correlation to the process of ovarian aging has expanded the potential for future research into the variation in ovarian aging in humans unexplained by age, which will greatly inform the understanding of female fecundity for epidemiological and clinical applications.

Clinical Applications for Ovarian Reserve Status

Anti-müllerian hormone has several clinical applications outside of its role in measuring ovarian aging and the decline in ovarian reserve over time as previously discussed. The hormone has also been used widely in infertility research. Perhaps the most established clinical use of AMH levels is before in vitro fertilization (IVF) because it can predict ovarian response to fertility medications (Grynnerup et al., 2012). Primarily, AMH predicts poor and hyper responses to fertility stimulation, which can improve the outcomes and safety of the procedure (Nelson et al., 2009; Grynnerup et al., 2012). AMH levels have also been preliminarily, in at least one study, demonstrated to be associated with live birth outcomes resulting from IVF, independent of

age (La Marca et al., 2010). Additional studies are “urgently awaited” to determine the relationship between initial AMH levels and outcomes related to fertility (Broer et al., 2014).

High AMH levels are associated with polycystic ovarian syndrome (PCOS), which affects between 5-10% of the female population, and represents another clinical application relevant to fecundity (Grynnerup et al., 2012). This finding may reflect the expression pattern of AMH levels in humans, with the current understanding being that the expression of AMH gradually disappears in follicles larger than 4 mm (Weenen et al., 2004). However, further research is particularly needed on the relationship between polycystic ovaries and follicle dynamics as these relate to AMH levels, as the role of androgens and metabolic disturbances may contextualize the observations of higher AMH levels associated with PCOS (Grynnerup et al., 2012).

The recommendations are still to be determined for screening the general population of individuals with ovaries for their ovarian reserve status based on AMH, given the lack of an established definition of normal variation (Tal & Seifer, 2017). Women with diminished ovarian reserve have also been observed to have higher rates of recurrent pregnancy loss in a recent systematic review, which has notable clinical relevance and suggests one potential biological pathway related to recurrent pregnancy loss (Bunnewell et al., 2020).

Primary ovarian insufficiency (POI) serves as another application of ovarian reserve. Also referred to as primary ovarian failure, it is a disorder characterized by infertility coupled with low estrogen levels, amenorrhea, and increased gonadotropin levels in women below the age of 40 years old (Visser et al., 2012). It is considered the result of the premature exhaustion of the follicle pool, though it can also be ascribed to follicular dysfunction. However, this disorder is considered distinct from natural menopause. The mechanisms implicated for POI include a

developmental reduction in the number of primordial follicles at birth, an accelerated rate of follicle recruitment, increased rates of destruction or atresia of follicles due to autoimmune antibodies each have the potential to lead to the disorder, among other dysfunctions in the follicle development process owed to genetic or other causes (Visser et al., 2012).

Similarly, the condition known as diminished ovarian reserve (DOR) is diagnosed by abnormal but not postmenopausal ovarian reserve testing and regular periods. This is distinct from POR/POI, which relates to postmenopausal levels of FSH and four months without menses (Pastore et al., 2018). The normal process of ovarian aging that typically occurs in the years prior to natural menopause is referred to as DOR, though if it is to occur prematurely it is considered pathological (Pastore et al., 2018). However, it is still notable that these definitions are not fully standardized amongst clinicians, and there remains some ambiguity as to what constitutes a pathologic state as it relates to ovarian reserve status. There has been a recent improvement in tools that measure ovarian reserve, but the spectrum of normal aging as compared to premature aging is not yet well understood (Pastore et al., 2018).

One other emerging term from the literature on ovarian reserve as it relates to its clinical applications is “functional ovarian reserve”, which attempts to distinguish functional versus non-functional follicles within the larger definition of total ovarian reserve (Iliodromiti et al., 2016). Functional ovarian reserve refers more specifically to the AFC rather than attempting to extrapolate to the total ovarian reserve, measured by primordial follicles, though the latter is difficult to capture as previously discussed (Pastore et al., 2018). Further complicating the relationship and distinction between the normal pace versus an abnormal pace of ovarian aging, as it relates to ovarian reserve, is whether the measurement of quantity reflects the level of quality in oocytes. Studies have suggested that the fecundity of women with lower ovarian

reserve remains similar to age-matched individuals with normal ovarian reserve (Baris et al., 2019). Therefore, the quantitative decline in ovarian reserve at an earlier age than typically observed may not necessarily reflect lower fecundity. However, as discussed, other studies have correlated ovarian reserve status to fecundity, and the relationship between ovarian reserve status and actual outcomes remains preliminary since oocyte quality cannot be easily measured.

Perhaps the largest problem that remains with research on ovarian reserve, as is related to the issue of the quantity and quality of oocytes, is the lack of normative data for the general population (Dillon & Gracia, 2013). Given that much of the literature to date on ovarian reserve status and AMH levels are measured within infertile samples, another issue remains to be seen on the appropriateness of applying findings among non-infertile populations. Conversely, it is likely inappropriate to utilize normative values based on population criteria for patients presenting with subfertility, infertility, or pathological ovarian states, though the findings can likely still be useful in clinical practice (Dillon & Gracia 2013). Ultimately, longitudinal, representative data is needed for clinicians, epidemiologists, and biological scientists to make a collective determination on the normal range of variation of ovarian aging and the normative decline in ovarian reserve.

Part III: Reproductive Ecology and Evolutionary Theory

Human Reproductive Ecology and Life History Theory

Human ovarian function and ovarian reserve status must be understood to be fundamentally intertwined with the evolution of our species, which only aids in our understanding of the underlying biology (Vitzthum, 2009; Ellison, 1990; Voland, 1998). Human ovarian follicle dynamics evolved in response to evolutionary pressures and processes, which therefore serve as a foundation for our present understanding of human ovarian biology. The

field of human reproductive ecology seeks to understand the complex relationship between variation in local environments to variation in reproductive traits (Vitzthum, 2009). Hormones should be considered agents of coordination between different biological systems, each of which has been shaped by the principles of evolution (Cox et al., 2016).

The study of reproductive endocrinology often fails to reconcile its central connection to the mechanisms of evolution (Cox et al., 2016). Fertility and reproductive effort remain at the forefront of the struggle to connect evolutionary theory to modern human biology, with the ovaries at the forefront of this relationship. Predicated by the aim to explain characteristics of human biology with an evolutionary lens, as well as the biology of other organisms, life history theory proposes the focus should be on the allocation of finite resources within the body. Life history theory is a school of thought within evolutionary biology that posits trade-offs between bodily systems and functions occur given that resources available for allocation are finite and ecologically grounded (Sterns, 2000). Resources used for one bodily function, under this theory, would not be allocated to another due to their finite availability. Total energy expenditure (TEE) encompasses both productive and basal energetic costs (Sterns,2000; Snodgrass, 2012). This extended version of the life-history theory is known as the study of energetics, with energy as essentially the unit of measure. The allocation of energy by the body allows human biologists to attempt to explain strategies of the human body to adapt to changing environments. Particularly, ovarian function, with its relationship to energy balance, has been implicated as an example of a life history tradeoff. The female body, considering insufficient availability of energy to meet the needs of both productive and basal metabolic functions, instead allocates energy away from reproduction through preventing ovulation (Snodgrass, 2012). This finding further suggests an

evolutionary benefit from suppressing reproductive effort, in consideration of energetic conditions, effectively trading off current reproduction for future reproduction (Ellison, 2003).

Additionally, the life history strategy of humans supports the quality of offspring over the total number of offspring, which relates back to the determinations of the body as to the ideal energetic conditions for reproduction (Hill 2008). Life history theory has also been utilized to explain stages of life within humans, from conception and beyond (Bogin & Smith, 1996; Ellison, 2017). However, considering ovarian reserve status as a chronic rather than an acute measure of ovarian function, and reproductive effort and potential, its connection to life history strategy and energetics remains uncertain. Further research on the connection between inflammation and the pace of ovarian aging across the lifespan may improve our understanding of human life history strategy.

Evolutionary Endocrinology

The field of evolutionary endocrinology remains promising given the endocrine system, its biomarkers, and function are rooted in evolutionary mechanisms. Perhaps the importance of the endocrine system within the paradigms of evolution and life history theory is best phrased by Worthman as follows: “Hormones, then, play crucial roles in affecting life-history processes in the day-to-day prioritization of resource allocation, as well as the long-term scheduling of growth, reproductive effort, and aging. Hence, measurement of hormones provides a window onto key determinants of ongoing function, adaptation, and differential well-being” (1999, p. 54). With this understanding in mind, the measurement of hormones to determine their role in the larger picture of energy allocation mobilizes evolutionary theory into the human body and bloodstream. Hormonal mechanisms within and connected to the endocrine system, are extremely sensitive to the internal and external environment of the organism, and subject to

flexibility through the life course (Finch & Rose, 1995). Further, the hypothalamic-pituitary-adrenal (HPA) axis plays a central role in modulating the endocrine system, as well as the greater neuroendocrine system. Human biologists have proposed its role to be fundamental in the regulation of both evolutionary and development determinants of function (Worthman, 2005). The timing of puberty and menopause, as well as the lifelong regulation of ovarian cycling, all occur in tandem with hormonal mechanisms (Vitzthum, 2009). Therefore, evolutionary endocrinology acknowledges the need to understand both the larger phenomenon that influences fecundity in women, as well as the micro-level control the endocrine system excerpts upon the body based on evolutionary life-history strategies. A full picture of the normal variability in ovarian steroid hormones, particularly in non-industrialized contexts, has yet to be observed (Vitzthum, 2009). This is also true of the AMH, alongside other biomarkers of ovarian reserve status. Evolutionary endocrinology, alongside life history theory grounded in an understanding of biological processes, serves as the lens to view the connection between immune function and ovarian reserve.

Part IV: Developmental Origins of Health and Disease

Early Environments and Adult Health

The "Developmental Origins of Health and Disease", originally termed "Fetal Origins Hypothesis", emerged from the association between slow growth during early life and coronary heart disease later in adulthood (Barker, 2002). Barker postulated that uterine environments shape adult health, in essence, "programming" the adult phenotype in utero, setting the stage for insulin resistance and subsequent heart disease later in life (Barker, 2002; Godfrey & Barker, 2001). Developmental plasticity, which refers to the "plastic" nature of human traits and genotypes, lies at the center of the relationship between early life growth and later life disease

(Lea et al., 2017; Sloboda et al., 2009). Human evolution has selected for offspring that have flexible expressions of phenotypes, to increase the chances of survival by better matching the environment it is born into (Lea et al., 2017). This flexibility and developmental plasticity are an important evolutionary trait in humans and has allowed our species to survive in some of the most extreme environments on Earth (Lea et al., 2017). However, if the early cues received by the fetus are incorrect, this can subsequently result in a mismatch between the programmed trait and the actual environment it is born into (Barker, 2002).

In the context of ovarian reserve, significant variability in the size of the primordial follicle pool during fetal development is seen (Wallace & Kelsey, 2010). Considering the high level of variability in the total ovarian reserve during fetal development and at birth, as well as variation in ovarian reserve biomarkers later in life, the Developmental Origins of Health and Disease (DOHaD) serves to explain this variation through the lens of developmental plasticity and uterine environment (Richardson et al., 2014). Though there likely remains a genetic component to ovarian reserve reached at birth, the question of other influences, including the maternal endocrine and nutritional environment and ecological factors, remains (Richardson et al., 2014). Particularly, building evidence suggests that maternal nutrient restriction and chemical exposures in utero, such as maternal smoking, may affect ovarian reserve, influencing the developmental trajectory of ovarian reserve in adulthood (Richardson et al., 2014).

The Fetal Environment and Age at Natural Menopause

The relationship between the fetal environment and early age at natural menopause has also been explored. The earlier timing of natural menopause, assumed to mechanistically occur through both the size of the initial follicle pool and the rate of follicle loss, has been associated with both low birth weight and high birth weight for gestational age (Tom et al., 2010). Research

also suggests that being born small for gestational age, premature birth and lower birth weight are associated with slightly higher odds of premature ovarian insufficiency (POI) (Sydsjo et al., 2020). Though natural menopause and the pathological condition of POI likely represent different biological mechanisms, it is still notable that the findings are consistent with one another. In the Sister Study, with 22,165 participants aged 35 to 59 at enrollment, earlier age at menopause, after controlling for age, race, and ethnicity, education, childhood family income, and smoking, was associated with in-utero diethylstilbestrol (DES) exposure, maternal pre-pregnancy diabetes, and low birth weight (Steiner et al., 2010). Maternal under or over nutrition, stress, and exposure to bisphenol A, phthalates and other endocrine disrupting chemicals remain as a promising explanation for the developmental establishment of total ovarian reserve in utero (Puttabyatappa & Padmanabhan, 2018). The size of the total ovarian reserve is hypothesized to influence age at natural menopause (Puttabyatappa & Padmanabhan, 2018).

Variation in Anti-Mullerian Hormone Levels

In the context of adult AMH levels, studies have identified differences in serum AMH levels by self-identified race and ethnicity (Bleil et al., 2015; Seifer et al., 2011). However, both studies were cross-sectional, making it difficult to ascertain the overall ovarian reserve trajectory of individuals across the life course. In the Bleil and colleagues' study, results suggested that self-identified African American women, cross-sectionally, had lower AMH levels at younger ages, but displayed a slower decrease in AMH at older ages, which may represent different paces of ovarian aging (2015). Further, in this study, self-identified Chinese and Latina women, as compared to self-identified white women, had lower AMH levels at all ages (Bleil et al., 2015). The latter study, a prospective cohort study on HIV-positive women, compared AMH levels among self-identified white, black, and Hispanic women and found statistically significant

differences between self-identified white and black women after controlling for age, BMI, HIV stats, and smoking, with self-identified black individuals having lower AMH levels (Seifer et al., 2011). Some studies have also reported that African American and Latina women have natural menopause approximately 2 years earlier than white women (Gold, 2011). However, this finding is complicated by disparities in age at menopause by socioeconomic status, which is deeply embedded with cultural and historical context as these relate to race and ethnicity, particularly in the United States, which may explain some inconsistent results reported in the age of natural menopause and race and ethnicity (Gold, 2011).

It is important to note, however, that these observed differences do not represent genetic differences given that racial and ethnic groups are socially constructed and without a biological basis (Smedley & Smedley, 2005). Rather, the embodiment of the social environment into the body and its biological processes would be the most plausible explanation for observed differences, through developmental programming and other mechanisms (Gravelee, 2009). Differences in other aspects of reproductive function, particularly regarding infertility treatment and reproductive assistive technology (ART) have also been observed between ethnic and racial groups, illustrating the importance of understanding the underlying biological mechanisms and context in which these outcomes exist (Huddleston et al., 2010). Begum and colleagues best support this assertion as it relates to AMH levels and ovarian reserve status in their study on the effects of migration on ovarian reserve among Bangladeshi women in the UK (Begum et al., 2016). The researchers found that Bangladeshi migrants that moved to the United Kingdom (UK) as children and European women had significantly larger age-related ovarian reserve compared to migrant Bangladeshis who moved to the UK as adults and Bangladeshi women still residing in Bangladesh (Begum et al., 2016). This pivotal study suggests that childhood development and

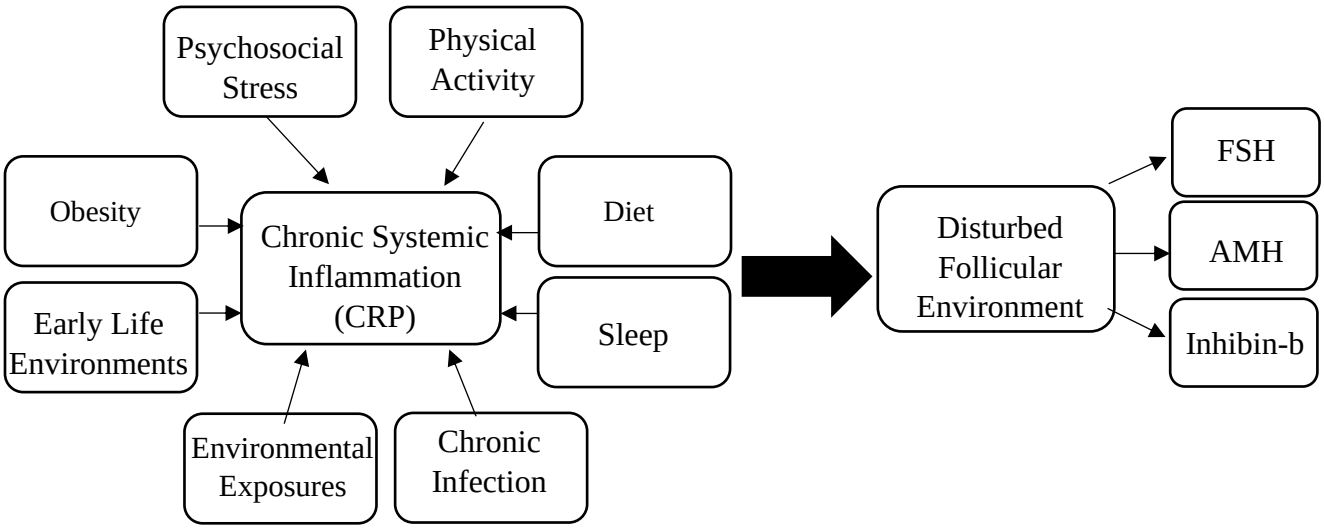
early life environments should be considered when observing variation in ovarian reserve status, particularly between ethnic groups (Begum et al., 2016). Similar research along this line in another geographical and social context has found that Maya ethnicity was associated with a higher likelihood of having undetectable AMH levels, as compared to non-Maya women, which is consistent with previous studies on the earlier age at natural menopause in Maya women (Kyweluk et al., 2018). Earlier age at menarche may also represent distinct reproductive trajectories and pacing, which are likely influenced by early life environments. Age at menarche has been demonstrated to have a statistically significant relationship with lower age-matched ovarian reserve as measured by AMH later in life (Bragg et al., 2012; Weghofer et al., 2013). However, this may simply be a marker of relatively "faster" reproductive pacing rather than representing a causal relationship.

Lifestyle Determinants of Earlier Age of Natural Menopause

Several other lifestyle factors and determinants have been associated earlier age at natural menopause outside of early life environments, including smoking, lower parity, and lower SES (Sun et al., 2012; Gold, 2011). Additionally, obesity and body size have been associated with lower serum AMH levels (Su et al., 2010). However, results have been somewhat inconsistent with obesity and socioeconomic status (Dolleman et al., 2013). Smoking and oral contraceptive use have been associated with lower AMH levels as well, but these observed effects may be reversible, though this is not yet well understood (Plante et al., 2011; Dolleman et al., 2013). There are also racial and ethnic differences in smoking and obesity rates, these differences may in part explain some of the variations observed in ovarian reserve (Tal & Seifer, 2013). Chronic, lifetime psychosocial stressors may also be related to lower ovarian reserve, though current stress was not related to markers of ovarian reserve (Pal, Bevilacqua, and Santoro 2010). Several genes

have been associated with a higher likelihood of experiencing ovarian pathologic states, such as POI, however, these do not explain the majority of cases based on current understandings (Schuh-Huerta et al., 2012; Fortuno & Labarta, 2014). It is also likely that this clinical and pathological state may have different mechanisms outside of normal ovarian aging, as previously mentioned. Overall, there is significant evidence to suggest that early life and childhood environment could explain the observed variation in age at natural menopause, ovarian reserve, and subsequent fertility outcomes, which may have implications for reproductive and somatic health alike (Cedars, 2013).

Figure 1. *Conceptual Framework for Chronic Systemic Inflammation on Ovarian Reserve Biomarkers*



AMH, anti-Mullerian hormone; FSH, follicle- stimulating hormone; CRP, C-reactive protein

CHAPTER 3: THE STUDY

METHODS

Study Design

The present analysis uses data from the Time to Conceive study, which was a prospective, time-to-pregnancy cohort study (2008-2016) in central North Carolina. The primary aim of the original study was to investigate the association between ovarian reserve and fecundability. Women in the Chapel Hill region of North Carolina who were intending to become pregnant were recruited through mass emails, introductory letters, and radio and web advertisements. Eligibility to participate in the study required participants to be between the ages of 30 and 44 and to have been trying to conceive naturally for less than 3 months (self-reported). Additionally, women who had a history of infertility, endometriosis, or polycystic ovarian syndrome, had a partner with a history of infertility, or were currently breastfeeding were excluded from the study. The total size of the Time to Conceive study was 843 women, with 778 women having CRP measurements. The final sample size for AMH was 690 women, for FSH, 654 women and for Inhibin-b, 603 women.

The study included a self-administered questionnaire, which included demographic data, reproductive history, contraceptive history, tobacco, and smoking history, among other lifestyle and behavioral factors. Participants were asked to schedule a study visit at the start of their next menses, following study entry. At the study visit, which took place on day 2, 3 or 4 of the menstrual cycle, participants provided a venous blood sample. Women that did not have a blood

sample were excluded from the current analyses. All women provided informed consent and all study activities were approved by the University of North Carolina IRB or the Duke University IRB. AMH, FSH, and inhibin-b were measured from serum samples which were stored at -38°C until analysis. Samples were shipped frozen to and analyzed at University of Southern California Reproductive Endocrinology Laboratory. The assay used were as follows FSH (Immulite Analyzer, Siemens, Deerfield, IL). Inhibin-b (ELISA, Ansh Labs), and AMH, (Ultrasensitive AMH ELISA, Ansh). Interassay coefficients of variation were 4% to 5% for FSH, 5% to 8% for inhibin-b, and 9% to 11% for AMH.

The covariates initially evaluated were period regularity, body mass index, parity, gravidity, race, age at cycle start, highest level of education, and birth control use in the last one to three months. The covariates initially evaluated were based on previous associations between demographic and lifestyle factors and inflammation or ovarian reserve (Plante et al., 2011; Dolleman et al., 2013; Tal & Seifer, 2013; Kushner et al., 2006). Specifically, period regularity was evaluated by the participant as a yes or no, and therefore categorical and dichotomous. Body mass index was evaluated based on the participants self-reported height and weight in inches and pounds, respectively. The values reported were converted to kilograms and meters, and then the weight in kilograms were divided by the height in meters squared to arrive at the body mass index value, which was measured continuously. Parity included the number of births the participant reported. Gravidity included the number of all pregnancies the participant reported, included those that did not end in birth. Race was self-reported and measured categorically. The categories included white, African American, Asian or Pacific Islander, Native American or Native Alaskan, Hispanic, and Other. Age at cycle start was measured continuously and self-reported. It is important to note that the original study this data is derived from did not

differentiate between race and ethnicity, and therefore the category “Hispanic”, which is more commonly considered an ethnicity for classification, is reported alongside the other groups. Highest level of education was self-reported and categorical, and included having completed some college, having competed a 4-year college degree, some graduate school, and having completed a graduate degree. Birth control use was self-reported for using it in the last one month, two months, or three months, and was categorical and dichotomous.

The stratified characteristics calculated included the women’s age at cycle start, body mass index, parity, gravidity, race, highest level of education, cigarette smoking history, and period regularity. current smoking, hormonal birth control in the past 3 months or less, hormonal birth control in the past 1 month or less, hormonal birth control in the past 1 year or less, race, highest level of education, and period regularity.

Statistical Analysis

We used frequencies, medians, and interquartile ranges to describe the levels of CRP, FSH, and AMH and mean and standard deviation for inhibin-b within levels of each covariate. Inhibin-b was the only biomarker that appeared to have normally distributed values based each biomarkers kurtosis. CRP serum levels were also divided into two groups, high CRP (>3 mg/L) and low CRP (<3 mg/L) because levels above 3 mg/L have been identified as a clinical risk factor for cardiovascular disease (Ridker, 2003a). The median (IQR) FSH and AMH levels and mean (SD) levels were calculated within CRP categories. Finally, CRP levels were divided into four risk groups: <1 mg/L, 1 mg/L to 3 mg/L, 3 mg/L to 10 mg/L, and >10 mg/L. Again, the median (IQR) FSH and AMH levels and mean (SD) inhibin-b levels were calculated within each CRP category.

Unadjusted Spearman's correlation coefficients and 95% confidence intervals were calculated for the continuous covariates, age, body mass index, parity, gravity, and CRP, on each untransformed ovarian reserve biomarker, FSH, AMH, and inhibin-b. The unadjusted and untransformed Spearman correlation coefficients were also calculated for FSH and AMH, AMH and inhibin-b, and FSH and inhibin-b. The analysis was performed to evaluate the consistency of the data with previous studies.

Histograms and quartile-quartile plots showed that FSH, AMH, and CRP were not normally distributed and therefore were natural log-transformed. Inhibin-b was normally distributed and therefore was not natural log-transformed. Multivariate linear regression was used to estimate the association between CRP and each biomarker of ovarian reserve, FSH, AMH, and inhibin-b, adjusting for participants' body mass index, age at cycle start, race, hormonal birth control use in the last one, two and three months, and smoking history. Covariates included were based on previous literature on the factors associated with differences in the values of the biomarkers ovarian reserve (Plante et al., 2011; Dolleman et al., 2013; Tal & Seifer, 2013). The residuals were examined to confirm these were normally distributed, including a good fit of the adjusted model.

All statistical analysis was conducted in Stata/IC Version 16.1.

RESULTS

Sample Characteristics and Summary of Stratified Biomarkers

Most study participants were between 31 and 35 years old at the start of the menstrual cycle in which blood was drawn, were in the normal range of BMI (58%), classified their race as white (77%), and reported never smoking (76%). Underweight BMI (<18.5 kg/m2) was the least

frequent category (3%). The least represented category for self-reported race was Native American or Native Alaskan (0.25%). There were few current smokers (2%). Most study participants reported the last use of hormonal contraceptive as more than three months (81%), reported having regular menses (87%), were nulliparous (51%) and have never given birth (66%). For the highest level of education, most study participants reported a professional or advanced degree (62%).

CRP. Median CRP tended to be higher in women of older age, with a higher BMI, who classified themselves as African American, or who reported either current or past smoking. Variability in CRP was greater, as evidenced by the wider IQR, with higher BMI, and was higher among Hispanic women. Those that classified themselves as Native American or Native Alaskan had the lowest median CRP followed by women who reported their race as white, followed by participants who classified themselves as "Hispanic". Median CRP was also higher among those that took hormonal contraceptive one month prior to the blood draw cycle starting, and lower for those that had not taken hormonal contraceptive for more than three months. However, the interquartile range remained larger for those that had taken hormonal contraceptives in the last one month and those that had not taken them for more than three months. Median CRP was lowest for those that reported one pregnancy, and highest for those that reported more than two pregnancies. Median CRP was generally higher for women who reported higher parity, but lower in those that reported one birth compared to those that reported less than 1. Median CRP was highest among those with some college or less and lower among those that had a professional or advanced degree. Median CRP was also higher among those reporting regular menstrual cycles, and lower among those that did not report regular menstrual cycles.

AMH. As expected, median AMH was lower for older participants, and the interquartile range was smaller. Median AMH tended to be higher for those who were overweight or underweight than those who were normal weight and were lowest for those who were obese. There was no clear pattern between AMH and BMI. Median AMH stratified by self-reported race was highest among those that classified themselves as Other, and lowest among those that classified themselves as Asian or Pacific Islander. Median AMH was also higher among past smokers, those that reported irregular periods, those with some college or less, those that reported taking hormonal contraceptives one month prior to the blood draw cycle, and those that had never been pregnant or given birth. Median AMH was lower among current smokers, those that reported taking hormonal contraceptives two months prior to the blood draw cycle starting, those that had a four-year college degree, and those that reported a regular menstrual cycle.

Inhibin-b. Mean inhibin-b levels and their standard deviation did not show a clear pattern of being higher or lower with age. For BMI, mean inhibin-b was highest for those who were underweight, and lowest for those who were obese, with the values for participants with BMIs in the overweight and normal range showing similar values for both the mean and standard deviation. For self-reported race, mean inhibin-b was highest among those that classified themselves as Native American or Native Alaskan and lowest among those that classified themselves as Hispanic. Mean inhibin-b was also higher for never smokers, those that had not taken hormonal contraceptive in the last three months, those that reported one pregnancy, those that reported never giving birth, higher among those with some graduate education, higher among those reporting regular menstrual cycles. Mean inhibin-b was also lower for current smokers, those that reported taking hormonal contraceptive two months prior to the blood draw

cycle starting, those that reported more than two pregnancies or births, and those reporting their highest level of education as some college or less.

FSH. Median FSH was generally higher at older ages; yet there was a larger interquartile range for participants between 36 and 44. For BMI, median FSH was highest among those who were classified as obese, and lowest for those who were classified as underweight. Those with a BMI classified as normal had higher median FSH levels than those with a BMI that was overweight. As well, for self-reported race, median FSH was highest among those that classified themselves as Native American or Native Alaskan, with those that classified themselves as Asian or Pacific Islander as the second highest and those that classified themselves as Hispanic having the lowest levels. Median FSH was higher for past smokers, higher among those that reported one pregnancy, those reporting two births, those with a four-year college degree and among those that reported regular menstrual cycles. Median FSH levels were lower among those that reported two pregnancies, those with some college or less, and those that reported irregular menstrual cycles.

The study demographics and the stratified biomarker medians and interquartile ranges by the sample characteristics are presented in detail in Table 1 to visualize the full results.

Table 1 Ovarian reserve biomarkers and CRP, stratified by participant characteristics

	CRP (mg/L)			AMH (ng/mL)			Inhibin-b (pg/mL)			FSH (mIU/mL)		
Variable	*N*	Median	IQR	*N*	Median	IQR	*N*	Mean	SD	*N*	Median	IQR
Age												
29-30	152	0.90	1.79	158	4.12	4.39	141	76.27	39.40	150	6.41	2.73
31-32	223	0.93	2.35	224	3.21	3.95	200	84.25	44.99	212	6.67	3.00
33-35	223	1.06	2.43	217	2.76	3.16	177	80.62	44.23	198	6.71	2.82
36-40	150	1.40	4.01	150	1.76	2.3	123	78.54	40.84	141	6.42	3.78
41-44	30	0.72	0.90	26	0.86	1.78	25	74.03	56.40	29	7.57	4.47
BMI												
Underweight	23	0.38	0.87	22	3.19	3.71	18	90.74	56.40	19	6.08	1.4
Normal	482	0.63	1.18	478	2.88	3.91	403	83.31	43.73	450	6.73	2.99
Overweight	157	1.75	2.46	118	3.17	3.38	140	80.76	44.04	147	6.29	2.71
Obese	115	4.25	7.95	156	2.44	3.56	104	65.81	35.30	113	6.87	3.16
Race												
African American	74	1.58	3.97	75	2.70	3.29	61	81.72	46.10	72	6.46	2.67
Native American/ Alaskan	2	0.29	0.02	2	2.69	4.68	2	133.99	16.32	2	8.23	2.70
Asian/Pacific Islander	53	1.16	1.94	54	2.51	4.13	50	80.62	40.90	51	7.11	3.19
White	601	0.95	2.08	596	2.88	3.68	512	79.89	43.47	559	6.62	3.06
Hispanic	18	1.00	4.32	18	2.99	2.82	15	77.89	37.98	16	6.17	2.44
Other	30	1.20	2.50	30	3.11	5.25	26	78.08	44.39	30	6.48	2.11
Smoking History												
Never	593	0.93	2.20	590	2.82	3.84	505	81.30	44.02	553	6.6	3.09
Current	14	2.07	3.39	14	1.49	4.46	14	64.03	43.73	14	6.89	5.12
Past	171	1.16	2.56	171	2.98	3.37	147	77.76	40.91	163	6.6	2.56
Months Since Hormonal Contraceptive Use												
One or Less	30	2.50	4.78	30	3.11	3.57	18	80.86	70.32	25	6.1	3.07
Two	21	0.96	1.37	20	1.75	2.81	18	70.18	37.47	20	7.56	2.62
Three	43	0.91	1.29	44	2.67	4.90	38	74.93	43.10	40	7.24	3.41
Three or More	684	0.93	2.28	680	2.88	3.63	591	80.88	42.54	644	6.54	2.89
Parity												
0	510	0.91	1.98	511	3.11	4.20	437	81.04	44.06	480	6.53	2.93
1	211	1.23	2.48	208	2.39	3.00	177	79.64	41.12	194	6.63	2.97
2	43	1.22	3.78	43	2.39	4.55	38	79.91	46.17	42	7.65	3.27
>2	14	2.50	4.32	13	2.45	3.32	14	59.81	41.13	14	6.57	1.63
Gravidity												
0	396	0.94	2.08	395	3.34	4.2	333	80.04	44.52	365	6.60	2.76
1	208	0.86	1.97	207	2.42	2.95	180	82.53	42.72	197	6.69	3.02
2	111	1.38	3.09	112	2.85	4.31	97	78.31	40.13	109	6.55	3.03
>2	63	1.63	2.89	61	2.33	2.12	56	76.43	44.64	59	6.62	3.57

Education Level												
Some College or Less	59	2.64	7.09	60	3.21	3.60	53	75.44	75.44	56	6.46	2.58
4-year College degree	163	1.43	3.39	165	2.28	3.30	146	75.68	75.68	158	6.80	2.59
Some Graduate	67	1.01	2.40	68	3.04	4.44	64	84.95	84.95	65	6.48	2.36
Professional/Advanced Degree	489	0.85	1.76	482	2.90	3.84	482	81.64	81.64	451	6.62	3.24
Period Regularity												
Yes	673	1.03	2.45	670	2.78	3.42	576	80.50	42.58	632	6.63	2.89
No	104	0.68	1.72	104	3.75	5.24	89	77.89	48.62	97	6.14	3.35

AMH, anti-Mullerian hormone; FSH, follicle- stimulating hormone; CRP, C-reactive protein; IRQ, interquartile range; SD, standard deviation

Bivariate Analysis Results

Bivariate analysis was supplemental and the results from Table 1, alongside the previous literature cited in the methods, were used to determine the appropriate adjustments for the multivariate model of CRP on AMH, FSH, and inhibin-b. Variables selected for bivariate analysis included current smoking, hormonal birth control in the past 3 months or less, hormonal birth control in the past 1 year or less, race, highest level of education, and period regularity. These are reported in Table 2 and described below.

Race was highly statistically significant for mean natural log-transformed CRP measurements between groups ($F = 2.99$; $p = 0.01$). Additionally, highest level of education was statistically significant for mean log-transformed CRP measurements between groups ($F = 6.64$; $p = <0.01$). Period regularity was statistically significant for mean natural log-transformed CRP measurements difference between groups ($t = -2.22$; $p = 0.03$. Period regularity was also statistically significant for mean natural log transformed AMH measurements difference between groups ($t = -2.5$; $p = 0.01$). There were no other significant associations between any the four biomarkers and smoking in the past or present, hormonal contraception usage in the last one year or three months.

Unadjusted Spearman's Coefficient Correlation Results

Spearman correlation analyses were conducted on each of the unadjusted, untransformed values of AMH, FSH, inhibin-b, and CRP for age, BMI, parity, and gravidity. The Spearman correlation coefficient for age and AMH was -0.32 and was highly statistically significant ($p = <0.01$). The Spearman correlation coefficients for age and the other three biomarkers were close to 0 and were not statistically significant. For BMI and inhibin-b, the Spearman correlation

coefficient was -0.17 (p = <0.01). For BMI and CRP, the Spearman correlation coefficient was 0.50 and was highly statistically significant (p = <0.01). Parity and AMH had a -0.18 value for the Spearman correlation coefficient (p = <0.01). Parity and CRP had 0.12 Spearman correlation coefficient (p = <0.01). Gravidity and AMH had a -0.17 Spearman correlation coefficient (p = <0.01). Gravidity and CRP had a 0.03 Spearman correlation coefficient (p = 0.03)

The unadjusted Spearman's coefficients and their 95% confidence intervals calculated to assess the relationship between CRP and the three markers of ovarian reserve, AMH, FSH, and Inhibin-b. The Spearman's correlation coefficient for CRP and inhibin-b was -0.20, with the 95% confidence interval -0.27 to -0.12 (p = <0.01). For CRP and AMH, the Spearman correlation coefficient was 0.02, with the 95% confidence interval -0.06 to 0.09 (p = 0.68). For CRP and FSH, the Spearman's correlation coefficient was -0.11, with the 95% confidence interval -0.18 to 0.03 (p = <0.01). For AMH and FSH, the Spearman's correlation coefficient was -0.32, with the 95% confidence interval -0.38 to -0.25 (p = < 0.01). For AMH and inhibin-b, the Spearman's correlation coefficient was 0.15, with the 95% confidence interval 0.08 to 0.23 (p = <0.01). For inhibin-b and FSH the Spearman's correlation coefficient was -0.09, with the 95% confidence interval -0.17 to 0.02 (p = 0.03).

Table 2. Spearman's Correlation Coefficients for Covariates				
Variables	AMH	FSH	Inhibin-b	CRP
Age Spearman's Coefficient p-value	 -0.32 <0.01*	 0.07 0.06	 -0.01 0.77	 0.07 0.07
BMI Spearman's Coefficient p-value	 -0.05 0.17	 -0.04 0.32	 -0.17 <0.01*	 0.50 <0.01*
Parity Spearman's Coefficient p-value	 -0.18 <0.01*	 0.02 0.63	 -0.06 0.18	 0.12 <0.01*
Gravidity Spearman's Coefficient p-value	 -0.17 <0.01*	 -0.01 0.77	 -0.03 0.48	 0.03 0.04*

BMI, Body mass index; AMH, anti-Mullerian hormone;
FSH, follicle- stimulating hormone; CRP, C-reactive protein

Table 3. Spearman's Correlation Coefficients (and 95% Confidence Intervals) between CRP and Ovarian Reserve Biomarkers			
Variable	AMH	FSH	Inhibin-b
CRP Spearman's Coefficient (95% CI) p-value	 0.02 (-0.06 to 0.09) 0.68	 -0.11 (-0.18 to -0.03) <0.01*	 -0.20 (-0.27 to -0.12) <0.001*
Inhibin-b Spearman's Coefficient (95% CI) p-value	 0.15 (0.08 to 0.23) <0.001*	 -0.09 (-0.17 to -0.02) 0.03*	 -
AMH Spearman's Coefficient (95% CI) p-value	 -	 -	 -
FSH Spearman's Coefficient (95% CI) p-value	 -0.32 (-0.38 to -0.25) <0.001*	 -	 -

AMH, anti-Mullerian hormone; FSH, follicle- stimulating hormone; CRP, C-reactive protein

Adjusted Multivariate Regression Results

For each outcome, an adjusted multivariate regression was conducted to assess the relationship between CRP and the three markers of ovarian reserve, AMH, FSH, and inhibin-b, adjusting for age at cycle start, smoking history, body mass index, and use of hormonal birth control in the last three months or less. CRP, FSH, and AMH were log transformed in each multivariate regression.

For the association between the predictor CRP on the outcome of AMH, the beta coefficient was 0.04, with a 95% confidence interval of -0.01 to 0.10 (p = 0.13). For FSH, the beta coefficient was -0.02, with a 95% confidence interval of -0.05 to 0 (p = 0.05). For inhibin-b, the beta coefficient was -3.07, with a 95% confidence interval of -5.92 to -0.21 (p = 0.04). Supplementary analysis removing the current smokers (*N*=14) found no significant difference in the beta coefficient results.

Table 4. Adjusted Associations between CRP and Ovarian Reserve Biomarkers[a]

Variables	AMH[b]	FSH[b]	Inhibin-b[c]
CRP[b]	N = 690	N = 654	N = 603
Regression Coefficient	0.04	-0.03	-3.07
(95% CI)	(-0.01 to 0.10)	(-0.05 to 0)	(-5.92 to -0.21)
P>|t|	0.13	0.05*	0.04*

[a]Adjusted for age, race, smoking history, BMI, and use of hormonal birth control in the last three months or less.
[b]Natural log-transformed
[c]Inhibin-b is not log-transformed.
AMH, anti-Mullerian hormone; FSH, follicle- stimulating hormone; CRP, C-reactive protein

CRP Subgroup Analysis

CRP was then dichotomized with the two groups representing values above or below 3 mg/L. T-tests were performed for mean difference between the higher versus lower group on each of the three ovarian reserve biomarkers. CRP serum levels were also grouped into four categories for ANOVA analysis: group 1 with levels below 1 mg/L, group 2 with levels between

1 mg/L and 3 mg/L, group 3 with levels between 3 mg/L and then group 4 with levels above 10 mg/L. Lastly, a final adjusted multivariate regression model was run with the four groups of CRP value ranges with group 1 serving as the reference group.

For the additional bivariate analysis for higher versus lower CRP serum levels did not find a statistically significant difference between the two groups for log transformed FSH or AMH levels. However, there was a statistically significant difference in mean serum inhibin-b levels between the two groups ($p = <0.01$). Additionally, the ANOVA results between the four subgroups of CRP also found a statistically significant difference between mean inhibin-b levels by group ($p=<0.01$). The ANOVA results for AMH and FSH were not statistically significant. These results can be viewed in Table 6 and inhibin-b levels by subgroup are presented in Figure 2.

For the supplementary adjusted multivariate regression model by the four CRP subgroups, with group 1 serving as the reference group, there were statistically significant differences between group 1, with values below 1 mg/L and the two highest groups. There were no statistically significant differences between group 1 and group 2.

For group 3, with CRP values between 3 mg/L and 10 mg/L, for the outcome of log transformed AMH, the beta coefficient was 0.24 with a 95% confidence interval of 0.02 to 0.46 ($p = 0.03$). Additionally, log transformed FSH, the beta coefficient was -0.07, with a 95% confidence interval of -0.17 to 0.03 ($p = 0.17$). For inhibin-b, the beta coefficient was -11.08, with a 95% confidence interval of -21.91 to -0.24 ($p = 0.05$).

For group 4, with CRP values above 10 mg/L, for the outcome of log transformed AMH, the beta coefficient was 0.32 with a 95% confidence interval of 0.03 to 0.62 ($p = 0.03$).

Additionally, for log transformed FSH, the beta coefficient was -0.13, with a 95% confidence interval of -0.27 to 0 (p = 0.06). For inhibin-b, the beta coefficient was -17.89, with a 95% confidence interval of -32.69 to -3.09 (p = 0.02).

Table 5. Bivariate Analysis for CRP Subgroups and Ovarian Reserve Biomarkers

Variables	AMH[a]	FSH[a]	Inhibin-b[b]
CRP Above or Below 3 mg/L Test Statistic p-value	 t = -0.89 p = 0.38	 t = 1.88 p = 0.06	 t = 4.17 p = <0.01*
CRP Group Risk Level Test Statistic p-value	 F = 0.52 p = 0.67	 F = 1.63 p = 0.18	 F = 7.91 p = <0.01*

[a]*Natural log-transformed*
[b]*Inhibin-b is not log-transformed.*
AMH, anti-Mullerian hormone; FSH, follicle- stimulating hormone; CRP, C-reactive protein

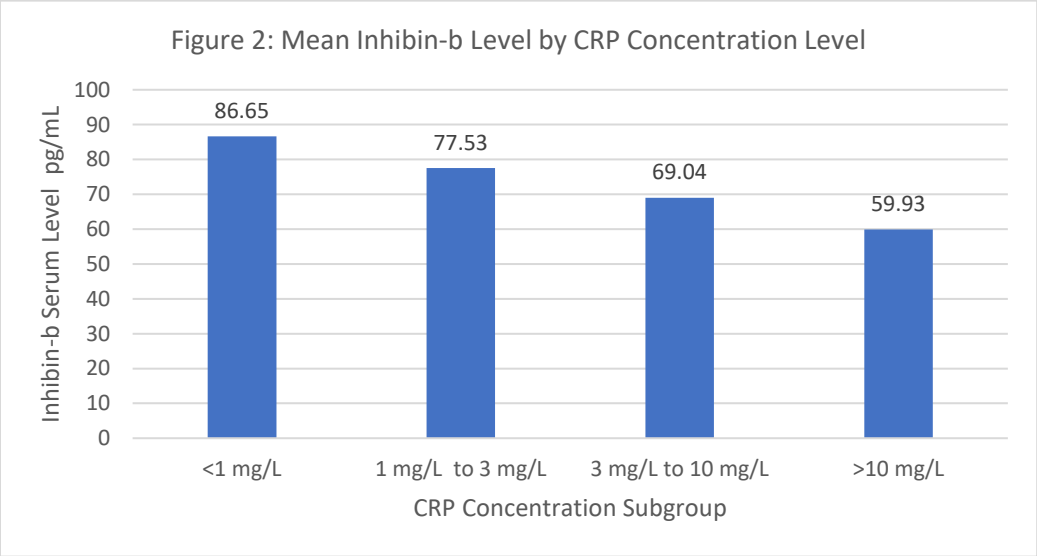


Table 6. Adjusted Association Between CRP Subgroup and Ovarian Reserve Biomarkers[a]			
Variables	AMH[b]	FSH[b]	Inhibin-b[c]
CRP 1 mg/L to 3 mg/L[d]	*N = 193*	*N= 194*	*N= 166*
Regression Coefficient	0.08	*-0.02*	*-4.81*
95% CI	-0.10 to 0.24	*-0.10 to 0.06*	*-13.35 to 3.73*
P>|t|	0.43	*0.65*	*0.27*
CRP 3 mg/L to 10 mg/L[d]	*N= 110*	*N= 101*	*N= 97*
Regression Coefficient	*0.24*	-0.07	*-11.08*
95% CI	*0.02 to 0.46*	-0.17 to 0.03	*-21.91 to -0.24*
P>|t|	*0.03**	0.17	*0.05**
CRP > 10mg/L[d]	*N= 50*	*N= 49*	*N= 45*
Regression Coefficient	*0.32*	-0.13	*-17.89*
95% CI	*0.03 to 0.62*	-0.27 to 0	*-32.69 to -3.09*
P>|t|	*0.03**	0.06	*0.02**

[a]*Adjusted for age, race, smoking history, BMI, and use of hormonal birth control in the last three months or less.*
[b]*Natural log-transformed*
[c]*Inhibin-b is not log-transformed.*
[d]*Compared to CRP below 1 mg/L (AMH: N = 338; FSH: N=319; Inhibin-b: N = 296)*
AMH, anti-Mullerian hormone; FSH, follicle- stimulating hormone; CRP, C-reactive protein

DISCUSSION

In the adjusted multivariate regression analysis with CRP analyzed continuously, CRP was not strongly associated with AMH. For every 1% increase in CRP, there was an associated 0.04% increase in AMH, but this relationship was not statistically significant. However, these results change if CRP is analyzed by subgroup, specifically when CRP higher than 3 mg/L, which represents the American Heart Association's (AHA) classification of high risk for cardiovascular events (Pearson et al., 2003; Ridker, 2003a; Ridker, 2003b). In the adjusted multivariate results with CRP values placed into four categories, rather than CRP analyzed as a continuous variable, found that AMH was statistically significantly higher in women with CRP between 3 mg/L to 10 mg/L and in women with CRP above 10 mg/L were compared to the group with CRP below 1 mg/L.

Therefore, these findings support previous research that has found a connection between lower ovarian reserve as measured through AMH and chronic autoimmune conditions, when CRP is above the 3 mg/L threshold (Cui et al., 2016; Freour et al., 2012). Cui and colleagues found that chronic pelvic inflammatory disease was associated with lower AMH levels, but not FSH levels (2016). Additionally, Freour and colleagues have reported that in women with Crohn's disease that are older than 30 years old, AMH levels were significantly lower, but not for women younger than 30 years old (2012). However, these studies did not directly measure CRP directly, unlike the present study. The connection between chronic inflammation and ovarian reserve, based on the difference in results between the two models, suggests that CRP may only influence AMH when CRP is above 3 mg/L. This would be consistent with findings that CRP above 3 mg/L is associated with higher health risk (Ridker, 2003b). Further, the mechanism regulating the ovarian aging process remains largely unobserved in human biology research. In one animal model performed by Lliberos and colleagues, in the ovaries of female mice, there was an association between inflammatory markers in the ovary and follicle depletion (2012). A similar study by Zhang and colleagues in female mice found signs of inflammaging in the mammalian ovary, with older ovaries showing increased signs of inflammation compared to younger ovaries (2020). It remains unclear if the findings from animal models suggesting a connection between chronic inflammation and the normal or accelerated ovarian aging process will be seen in humans (Lliberos et al., 2021; Zhang et al., 2019).

The second key finding is that CRP is associated with a significant difference in FSH in the multivariate regression model. For every 1% increase in CRP, there was an associated 0.02% decrease in FSH that was statistically significant ($p = 0.05$). However, these results change if CRP is analyzed by subgroup. When CRP is higher than 3 mg/L, FSH was not significantly

different between the lowest CRP group and the remaining three groups. The second model is consistent with the previous finding by Cui and colleagues (2016) that FSH was not associated with significant differences in those with pelvic inflammatory disease, but again there does not appear to be a similar study that looks at CRP directly. FSH is associated with ovarian aging as follicles become increasingly desensitized to it and therefore larger and larger amounts are released (Kim et al., 2017). It is unclear, given the slight effect of FSH, whether CRP has a biologically significant effect, particularly for ovarian aging. Given the association between follicle development declining with increasing levels of inflammatory markers in mice ovaries, however, one would expect lower levels of age-adjusted FSH associated with higher levels of inflammation, if there were an independent effect beyond age alone (Zhang et al., 2020). It remains possible that the rate of reproductive aging could differ from chronological age, given the variation in ovarian reserve levels that have been documented (Dolleman et al., 2013). That considered, the relationship between CRP and FSH has not been observed in a healthy human population to date based on currently published literature, and thus these results remain preliminary.

The third key finding is that CRP was significantly associated with a decrease in inhibin-b in the multivariate regression model. For every 1% increase in CRP, there was an associated 0.03 pg/mL decrease in inhibin-b, and this relationship was statistically significant ($p = 0.04$). Additionally, the adjusted multivariate results with CRP values placed into four categories found that inhibin-b was statistically significantly higher in women with CRP between 3 mg/L to 10 mg/L and in women with CRP above 10 mg/L were compared to the group with CRP below 1 mg/L. Inhibin-b is a product of follicles that have diameters smaller than 2 mm and appears to have an inverse relationship to FSH during the follicular phase of the menstrual cycle (Honour,

2017; Robertson, 2012). It is thought that inhibin-b levels decline over the reproductive lifespan, which leads to increasing FSH with reproductive age (Robertson, 2012). However, inhibin-b is still considered a poor marker of ovarian reserve compared to AMH overall (Honour, 2017). It is still notable that one of the earliest signs of ovarian aging hormonally is the increase in FSH resulting from decreasing levels of inhibin-b, though this is still debated in the literature on the biochemistry of menopause (Hall, 2020). Higher inhibin-b levels have also been previously associated with decreased risk of a short luteal phase (Pfister et al., 2019). Inhibin-b further may represent antral follicle potential and development and play a role in a normal luteal phase, though the connection between inhibin-b and successful conception remains unclear (Pfister et al., 2019). The relationship between CRP and inhibin-b has not been previously observed in a healthy population, based on a review of current literature.

There were several additional findings in the bivariate analysis and descriptive analysis. The results from the bivariate analyses suggest that hormonal contraceptive use in the last month is significantly associated with serum CRP levels and serum AMH levels. The use of oral contraceptives has been previously documented to increase serum CRP levels, but it is unclear what the mechanism of this relationship is (Sørensen et al., 2014). Additionally, it is well-documented that oral contraceptive use decreases AMH levels (Dólleman et al., 2013). Smoking was not associated with CRP or ovarian reserve measures, which is an unexpected finding given previous literature though this lack of association may stem from the relatively small number of smokers participating in the current study (Dólleman et al., 2013). Lastly, the association between race and CRP was statistically significant, suggesting that there may be a relationship between the lived experience of race and chronic inflammation response, which could be related to adult or early life environments (Kuzawa and Sweet, 2008; McDade, 2012). Education level

was additionally associated with CRP, but it is unclear if this would relate to other covariates rather than an effect on itself.

There were also a few notable findings from the CRP subgroup bivariate analysis. While FSH and AMH were not statistically different when compared between the two groups of below or above 3 mg/L, inhibin-b was highly statistically significant ($p = <0.01$). The ANOVA results between the four subgroups of CRP further support this relationship, with inhibin-b presenting with a significant p-value ($p= <0.01$). The finding that inhibin-b is associated with a statistically significant difference in its mean value by CRP value was unexpected given it is the only ovarian reserve biomarker that displayed this. Additionally, in data visualization by CRP subgroup, as presented in Figure 2, there appears to be a dose-response relationship with inhibin-b clearly declining as CRP levels increase. The biological explanation for this observed relationship remains unclear with increasing CRP levels being associated with decreasing inhibin-b levels. Inhibin-b represents an inhibitor of FSH production, which would suggest that lower levels of inhibin-b would be associated with higher levels of FSH (Robertson, 2012). However, further research is needed to understand how inhibin-b levels specifically change over the life course in relation to ovarian aging. It also remains possible that the sensitivity of the body to reproductive hormones changes over the life course (Kim et al., 2017). Decreasing sensitivity is seen with FSH, which may explain why inhibin-b levels are lower given the decreased need to regulate FSH.

Overall, the results from the adjusted multivariate regression with CRP placed into four categories suggests there was a significant difference in the regression coefficient between the lowest group of CRP values and the two highest groups for both AMH and inhibin-b, but not for FSH. However, the second-lowest group did not have a significantly different regression

coefficient for any of the ovarian reserve biomarkers. The second-lowest CRP concentration group may not have significantly differed from the lowest CRP concentration group because there may not be a significant difference biologically for values of CRP lower than 3 mg/L. Therefore, levels below 3 mg/L could show similar tendencies for their association to ovarian reserve biomarkers. The difference between the significance of the association between CRP and AMH in the two models suggests that when CRP levels were above 3 mg/L potentially have a biologically significant effect and threshold effect, rather than CRP at any level analyzed continuously.

LIMITATIONS AND FUTURE DIRECTIONS

The Time to Conceive study may be limited in its generalizability given the sample characteristics, as they largely represent a college-educated, white population between the ages of 30 and 44. Further, given the study aimed at assessing time to conception by design, it was limited to women attempting pregnancy or soon to be attempting pregnancy at the time of enrollment, which has the potential to create sample bias (Weinburg et al., 1994). However, the study remains valuable in its connection between fecundability, fertility, and ovarian reserve biomarkers, particularly given the lack of previous research in this area prior to its initiation. Future research interested in the variability in ovarian aging may benefit from expanding the study population to individuals with ovaries regardless of pregnancy attempt status to better understand the relationship between ovarian reserve biomarkers and inflammation. This study design would also serve as a point of comparison for the Time to Conceive findings. Additionally, longitudinal data recording systemic, circulating inflammatory cytokines in the body, and specifically in the intra-ovarian environment over the reproductive life span in humans, would be useful for understanding the micro-environment of the ovary. It remains

largely unanswered whether the microenvironment of the ovary may differ from the larger bodily environment and it remains unclear the extent to which ovarian function is "buffered" from systemic inflammation in the body or if it is directly impacted by it (Clancy et al. 2013).

For biomarkers in the Time to Conceive study, these were only measured once, which has its limitations due to inter-cycle and intra-cycle variation. Though the biomarkers AMH and CRP are relatively stable across the menstrual cycle and all hormones were collected one time on day 2, 3, or 4 of the menstrual cycle for study participants, multiple points of collection across different menstrual cycles may be more representative of the true variability of the four biomarkers (La Marca et al., 2013; Wander et al., 2008). Additional immune biomarkers may be useful in future studies on the connection between ovarian aging and immune function.

Finally, data on environmental exposures, particularly known disruptors of ovarian and endocrine function, as well as a standardized measure of socioeconomic status may have complemented the study design and serves as an avenue for future research. The inclusion of factors such as psychosocial stress, socioeconomic status, dietary habits, and early life environments likely would enrich our understanding of the relationship between chronic inflammation and ovarian reserve biomarkers, or the lack thereof. The Time to Conceive had several limitations due to its study population and original research questions, and though these results are illuminating, they do not necessarily represent the larger population.

CONCLUSION

Overall, these findings suggest a slight, negative relationship between FSH and inhibin-b and CRP that was statistically significant, but not AMH in this sample of women attempting pregnancy in central North Carolina, when CRP is analyzed continuously. However, when CRP

is above 3 mg/L and compared to CRP below 1 mg/L, these findings suggest a slight negative between CRP and inhibin-b and a slight positive relationship between CRP and AMH. Given the breadth of ongoing research into the predictors of low ovarian reserve in humans, this project sought to explain variability in AMH, FSH, and inhibin-b by CRP. Life history theory would theorize that disruption to energy balance would lead to trade-offs between reproductive function and immune function. Further, reproductive effort may be downregulated if ecological conditions are not favorable, and it is not well-understood how this might unfold over the life course. The findings support the possibility of a relationship between inflammatory compounds in serum and ovarian aging in humans as demonstrated in female mice (Lliberos et al., 2021).

Future research should explore the relationship between chronic inflammation and ovarian reserve through a biocultural and socio-ecological framework in a general population of women regardless of pregnancy attempt status.

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